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Identification of Residues and Domains of Raf Important for Function *in Vivo* and *in Vitro**

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Random mutagenesis and genetic screens for impaired Raf function in *Caenorhabditis elegans* were used to identify six loss-of-function alleles of *lin-45 raf* that result in a substitution of a single amino acid. The mutations were classified as weak, intermediate, and strong based on phenotypic severity. We engineered these mutations into the homologous residues of vertebrate Raf-1 and analyzed the mutant proteins for their underlying biochemical defects. Surprisingly, phenotype strength did not correlate with the catalytic activity of the mutant proteins. Amino acid substitutions Val-589 and Ser-619 severely compromised Raf kinase activity, yet these mutants displayed weak phenotypes in the genetic screen. Interestingly, this is because these mutant Raf proteins efficiently activate the MAPK (mitogen-activated protein kinase) cascade in living cells, a result that may inform the analysis of knockout mice. Equally intriguing was the observation that mutant proteins with non-functional Ras-binding domains, and thereby deficient in Ras-mediated membrane recruitment, displayed only intermediate strength phenotypes. This confirms that secondary mechanisms exist to couple Ras to Raf *in vivo*. The strongest phenotype in the genetic screens was displayed by a S508N mutation that again did not correlate with a significant loss of kinase activity or membrane recruitment by oncogenic Ras in biochemical assays. Ser-508 lies within the Raf-1 activation loop, and mutation of this residue in Raf-1 and the equivalent Ser-615 in B-Raf revealed that this residue regulates Raf binding to MEK. Further characterization revealed that in response to activation by epidermal growth factor, the Raf-S508N mutant protein displayed both reduced catalytic activity and aberrant activation kinetics: characteristics that may explain the *C. elegans* phenotype.

The importance of the Ras signaling pathway in the regulation of cellular proliferation, differentiation, and survival has been well established using a variety of biochemical and genetic systems. One of the main Ras effectors is Raf, a serine/threonine kinase that is evolutionarily conserved in higher eukaryotes. Raf is a critical part of a signaling cascade that connects cell-surface receptors with regulatory events within the cell (1–4). Activated Raf proteins phosphorylate MEKs[1] on two serine residues, which in turn phosphorylate and activate ERKs (5–7).

Raf is localized to the cytoplasm as an inactive multi-protein complex. The activation of eukaryotic endogenous Raf molecules is dependent on relieving the interaction between the N-terminal regulatory and C-terminal kinase domain of the Raf molecule (8, 9). The initial event in Raf activation is the recruitment of Raf from the cytosol to the plasma membrane through a high affinity interaction between the switch 1 region of activated Ras-GTP and the N-terminal minimal Ras-binding domain of Raf (Raf RBD) (10–14). This step is critical for Raf activation as point mutations within Ras or Raf that disrupt this interaction block Raf activation (15, 16). However, the interaction between Ras and Raf alone is not sufficient for full Raf activation, because Ras-GTP cannot activate Raf unless Ras-GTP is membrane bound (12). Full Raf activation involves interaction of the Raf cysteine-rich domain (Raf CRD) with Ras and membrane phospholipids, dephosphorylation of specific Raf serine residues, and a complex series of phosphorylation events at serine, tyrosine, and threonine residues.

Several proteins bind to Raf and regulate its activity. The chaperone proteins Hsp90 and Cdc37 bind to and stabilize mammalian Raf proteins, holding them in a conformation permissive for recruitment by activated Ras (17, 18). Disruption of the Raf·chaperone complex *in vivo* reduces the half-life of Raf and abrogates Ras-dependent membrane recruitment (19, 20). Raf also binds to dimerized 14–3–3 at two phosphorylated serine residues, Ser-259 and Ser-621 (21). 14–3–3 also binds to the Raf CRD (22), an interaction that serves to stabilize the Raf·14–3–3 complex (23). 14–3–3 binding to cytosolic Raf maintains Raf in an inactive conformation permissive for Ras recruitment. Recruitment of Raf to the plasma membrane destabilizes the interaction of 14–3–3 with the N terminus, which allows phosphatases PP1 and PP2A to dephosphorylate Ser-259 thus removing 14–3–3 and allowing full Raf activation (24–27).

In mammals there are three Raf isoforms: A-Raf, B-Raf, and Raf-1 (C-Raf). Transgenic experiments indicate that the different Raf isoforms are non-redundant (28–33). A-Raf knockout mice are viable but suffer intestinal and/or neurological defects

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[1] The abbreviations used are: MEK, MAPK/extracellular signal-regulated kinase kinase; MAPK, mitogen-activated protein kinase; EGF, epidermal growth factor; ERK, extracellular signal-regulated kinase; RBD, Ras-binding domain; CRD, cysteine-rich domain; GFP, green fluorescent protein; BHK, baby hamster kidney cells.

depending on genetic background (30). B-Raf knockout mice have defects in neuroepithelial differentiation and the maintenance of endothelial cell viability and die *in utero* at 10–13 days post coitum due to vascular hemorrhage (31). Raf-1 knockout mice are anemic and die *in utero* or shortly after birth, with vascular defects in the yolk sac and placenta as well as an increase in the number of apoptotic cells throughout the embryo (28, 29, 32). Intriguingly, Raf-1 Y340F/Y341F "knockin" mice, which express the kinase-inactive RafFF mutant in place of wild-type Raf-1, survive to adulthood with no detectable phenotype (28). One conclusion from this study is that Raf-1 kinase function may not be required for normal mouse development. Instead, Raf-1 could play an anti-apoptotic role during development via a mechanism that is independent of its kinase function, perhaps by an interaction with the pro-apoptotic, stress activated protein kinase apoptosis signal-regulating kinase 1 (34).

Investigation as to whether Raf-1 kinase activity is critical for Raf-1 biological function is hampered in mammalian systems due to the overlapping activities of different Raf isoforms (33). Invertebrates have only a single Raf isoform (D-Raf in *Drosophila* and LIN-45 in *C. elegans*). In *C. elegans*, signaling pathways that involve Raf are used multiple times during development to control a variety of cell fate decisions (2). These signaling pathways have been characterized most extensively during the formation of the hermaphrodite vulva, a specialized epithelial structure used for egg laying (35). A mutation that reduces the activity of a gene in the Raf signaling pathway generates a vulvaless (Vul) phenotype. By contrast, a mutation that results in constitutive activity of one of these genes results in a multivulva (Muv) phenotype. Thus, vulva formation serves as an easily visualized readout of the activity of the Ras/Raf/MEK/ERK-signaling pathway. This signaling pathway regulates additional cell fate decisions including differentiation of the excretory cell, which is necessary for larval viability, and progression of germ cells through pachytene stage, which is necessary for fertility (36, 37). As there is only a single Raf isoform, genetic analysis in *C. elegans* is ideally suited to examine the functional significance of Raf within a physiological context.

To characterize the function of the Raf gene, we used random chemical mutagenesis and genetic screens for worms with developmental defects to identify a collection of *lin-45 raf* alleles (38). The molecular lesions in these alleles were identified, and the mutations were classified based on phenotypic severity; they ranged from weak loss-of-function mutations that cause mild defects to strong or complete loss-of-function mutations that cause severe phenotypes. Six of the alleles contain missense mutations that result in substitution of a single amino acid. To understand the biochemical defects caused by these mutations, we engineered them into homologous residues of vertebrate Raf-1 and characterized the biochemical properties of the mutant proteins in vertebrate cells. These assays included measurements of basal kinase activity, Ras-stimulated kinase activity, membrane recruitment, and binding to 14–3–3, Hsp90, Cdc37, and MEK. Our analysis identifies functionally significant residues in the Ras-binding domain, protein kinase domain as well as MEK and 14–3–3-binding domains and demonstrates the necessity of these residues and domains for Raf activity in an animal. An interesting observation that mirrors the Raf-1 knockout mice studies recently published is that *in vitro* Raf kinase activity does not necessarily correlate to the *in vivo* function of the Raf protein. The significance of this apparently paradoxical observation is discussed.

MATERIALS AND METHODS

Plasmids and Mutagenesis—Raf mutant constructs were generated using the QuikChange site-directed mutagenesis kit (Stratagene) using a human Raf-1 or B-Raf clone tagged with a FLAG or myc epitope. All constructs were sequenced prior to use. EXV-K-RasG12V, EXV-FLAG-Raf, EXV-FLAG-RafDD, and EXV-FLAG-RafCAAX have all been previously described (39). Raf-GFP was constructed by subcloning the Raf-1 cDNA into pEGFP-N3 (Clontech).

Cell Culture and Antibodies—COS cells and baby hamster kidney cells (BHK) were grown and maintained in HEPES-buffered Dulbecco's modified Eagle's medium containing 10% donor calf serum as described previously (39). Mouse monoclonal antibodies, anti-Raf-1, Hsp90, and Cdc37, were obtained from Transduction Laboratories, and anti-FLAG from Eastman Kodak Co. Polyclonal anti-14–3–3 antibody was obtained from Santa Cruz Biotechnology, Inc. Anti-Ras rat monoclonals (Y13–259 and Y13–238) were made from hybridomas acquired from the American Type Culture Collection. Polyclonal GFP antibody was obtained from I.A. Prior (University of Queensland) and monoclonal GFP antibody from Roche Applied Science. Phospho-MEK polyclonal and phospho-ERK monoclonal antibodies were purchased from New England Biolabs. Polyclonal MEK-1/2 (New England Biolabs) and ERK-1 polyclonal (Santa Cruz) were used as input control antibodies where indicated.

Cell Transfection and Immunofluorescence—COS cells were electroporated as described previously (40). After 54 h cells were switched to serum-free medium and incubated for a further 18 h before harvesting. BHK cells were seeded onto coverslips for immunofluorescence or 10-cm dishes for biochemical assays, and transfected using LipofectAMINE (Invitrogen) according to the manufacturer's instructions. Cells on 10-cm dishes were maintained in serum-free Dulbecco's modified Eagle's medium for 16 h after lipofection before being harvested. Cells were washed and scraped on ice into 0.5 ml of buffer A (10 mM Tris-HCl, pH7.5, 25 mM NaF, 5 mM $MgCl_2$, 1 mM EGTA, 1 mM dithiothreitol, 100 μM $NaVO_4$). After 10 min on ice, cells were passed 25× through a 23-gauge needle and the nuclei removed by low speed centrifugation. Post-nuclear supernatants were spun at 100,000 × *g*. The supernatant (S100) was removed, and the sedimented fraction (P100) was rinsed and sonicated for 5 min in 100 μl of ice-cold buffer A. The S100 fraction and resuspended P100 fractions were snap-frozen and stored at −70 °C in aliquots after measuring protein content by the Bradford reaction. Cells on coverslips were fixed in 4% paraformaldehyde 24 h after lipofection. The coverslips were washed for 10 min in phosphate-buffered saline, permeabilized with 0.2% Triton X-100 in phosphate-buffered saline, and blocked in 3% bovine serum albumin in phosphate-buffered saline. The primary antibody Y13–238 (Ras) was diluted in blocking buffer at a 1:2 to 1:30 dilution and the secondary antibody, anti-rat CY-3, used at 1:300 dilution. Raf-GFP was visualized by direct fluorescence.

Western Blotting—Expression and subcellular localization of ectopically expressed proteins were determined by immunoblotting. Cellular fractions, normalized for protein content, were resolved on SDS-PAGE gels and transferred to polyvinylidene difluoride membranes using semi-dry transfer. The membranes were probed with anti-Raf-1, Y13–259 for Ras, or anti-14–3–3, anti-Hsp90, anti-CDC37, or anti-GFP monoclonal and polyclonal antibodies as appropriate, then developed using horseradish peroxidase-conjugated secondary antibodies and enhanced chemiluminescence. Where indicated immunoblots were quantified by phosphorimaging (Bio-Rad).

Raf Kinase Assays—P100 aliquots of transfected cells were normalized for protein content and assayed for Raf activity using a two-stage coupled MEK/ERK assay with phosphorylation of myelin basic protein as readout (39). For assays of cytosolic Raf proteins, S100 fractions were normalized for Raf content and Raf proteins immunoprecipitated with M2 anti-FLAG monoclonal or anti-GFP polyclonal antibodies as previously described (23). Immunoprecipitates were then assayed for Raf activity as above. After the kinase assays, immunoprecipitates were taken up in SDS-PAGE sample buffer, resolved by SDS-PAGE, and immunoblotted.

RESULTS

Identification and Molecular Characterization of lin-45 raf Alleles—Alleles of *lin-45 raf* were identified by conducting genetic screens for *C. elegans* hermaphrodites with defective vulval development or sterility. To investigate how these mutations affect the activity of *lin-45* and the role of *lin-45* during development, the phenotypes of these mutants were characterized extensively (38). Based on these findings, the alleles were

TABLE I

lin-45 mutations cause larval lethality, sterility, and abnormal vulva formation

Twelve *lin-45* alleles were identified using random chemical mutagenesis and screens for abnormal vulval development or sterility. This collection includes six different missense mutations that result in single amino acid substitution: three alleles contain nonsense mutations (described in Ref. 38). *lin-45* mutations can be arranged in an allelic series that corresponds to an increasingly severe loss of LIN-45 activity. The *oz201* mutation appears to cause a complete loss of LIN-45 activity. Because *oz201* causes completely penetrant sterility, *oz201* homozygous mutants were derived from *oz201*/+ hermaphrodites. The other mutant strains are fertile, and homozygous mutants were derived from homozygous mutant hermaphrodites.

Genotype	LIN-45 mutation	Raf-1 mutation	Larval lethal[a]	Abnormal vulva[b]	Sterile[b]	Classification[c]
			%	%	%	
Wild-type			0	0	0	
n1925	R108W	H79W	1	1	0	W
n1924	I726F	V589F	5	0	1	W
n2520	S754F	S619F	0	0	0	W
n2018	P92S	P63S	76	24	1	I
n2506	R118W	R89W	86	93	3	I
oz201	S645N	S508N	55	100	100	S

[a] The percentage of hatched eggs judged to be homozygous mutants that generated dead larvae; most dead larvae displayed rigid, rod-like morphology.

[b] The percentage of all adult hermaphrodites judged to be homozygous mutants that displayed a severe egg-laying defect, no discernable vulva, or a protruding vulva (abnormal vulva) or that generated no larval progeny (sterile). A more detailed genetic analysis of the mutants is displayed in Hsu *et al.* (38).

[c] W, weak; I, intermediate; and S, strong.

arranged in a series of increasing severity that is likely to correspond to an increasing loss of *lin-45* activity; the series is the same whether larval lethality, vulval formation, or sterility are considered (Ref. 38 and Table I).

The Effect of lin-45 Loss-of-function Mutations on Ras Mediated Raf-1 Activation—To elucidate the biochemical basis of the inactivating mutations in *lin-45*, we initially generated the loss-of-function point mutations in *lin-45*. However, when expressed in mammalian cell lines the LIN-45 protein was catalytically inactive. We therefore turned to the well characterized model system of Ras-dependent Raf-1 activation in COS cells. K-Ras was used for these experiments because this mammalian Ras isoform most closely resembles *C. elegans let-60 ras*. Amino acid substitutions were introduced into FLAG-Raf (Raf-1 with an N-terminal FLAG epitope tag) at residues corresponding to the *lin-45* loss-of-function point mutations identified in the genetic screens (Table I and Fig. 1). Briefly, the *lin-45* mutations P92S, R108W, R118W, S645N, I726F, and S754F were introduced at the homologous Raf-1 residues P63S, H79W, R89W, S508N, V589F, and S619F, respectively. Wild-type and mutated FLAG-Raf-1 constructs were co-expressed with constitutively active RasG12V in COS cells. Membrane fractions of these cells were normalized for Raf-1 content, and Raf-1 kinase activity was measured in a coupled MEK/ERK assay. Fig. 2 shows that all of the *lin-45* point mutations, except H79W, profoundly abrogated K-Ras-dependent Raf-1 activation. Raf-1 mutants resistant to RasG12V activation may be deficient in membrane recruitment or refractory to later membrane activation events. To examine Ras-dependent plasma membrane recruitment, the *lin-45* point mutations were introduced into Raf-GFP (Raf-1 with a C-terminal GFP epitope tag). BHK cells were then co-transfected with the Raf-GFP constructs and RasG12V. Plasma membrane recruitment was assessed using confocal microscopy. Fig. 3 shows that Raf-GFP plasma membrane recruitment was abrogated by the two RBD point mutations P63S and R89W but was unaffected by the H79W substitution. None of the mutations outside of the Raf RBD compromised Raf-GFP membrane recruitment.

Effect of lin-45 Loss-of-function Mutations on Raf-1 Basal Kinase Activity and Associated Proteins—We next examined the effect of the *lin-45* point mutations on Raf-1 basal kinase activity and interaction with known associated proteins. FLAG-Raf and Raf-GFP constructs containing *lin-45* point mutations were expressed equivalently in COS cells and anti-FLAG or anti-GFP immunoprecipitates prepared from the cytosolic S100 fraction. Basal Raf kinase activity in immunoprecipitates normalized for Raf-1 protein was measured in a coupled MEK/ERK assay. The results in Fig. 4 show that point mutations within the Raf RBD, P63S, H79W, and R89W, had no effect on Raf-1 basal kinase activity. In contrast, point mutations within the Raf kinase domain, S508N and V589F, and a point mutation directly adjacent to the COOH-terminal 14–3–3 binding motif (S619F) all markedly reduced basal kinase activity. Immunoprecipitates were then blotted for 14–3–3, Cdc37, and Hsp90, which are important co-factors for Raf-1 activation. (FLAG immunoprecipitates could not be blotted for Cdc37, or GFP immunoprecipitates for 14–3–3, due to secondary antibody species cross-reactivity.) The mutations P63S, V589F, and S619F severely reduced the association of 14–3–3 with Raf-1, but no mutation had any significant effect on Hsp90 or Cdc37 interactions. Thus reduced 14–3–3 association has a minimal effect on Cdc37 or Hsp90 interactions with Raf-1.

Basal Raf-1 kinase activity could be reduced because of increased interaction between the N-terminal regulatory and catalytic domains, or structural disruption of the Raf-1 kinase domain. Replacement of tyrosines 340 and 341 with aspartic acid partially relieves the negative regulation of Raf-1 by the regulatory N terminus (41), allowing us to discriminate between these possibilities. We therefore introduced the *lin-45* mutations into FLAG-Raf Y340D/Y341D (RafDD), and measured the Raf-1 kinase activity of anti-FLAG immunoprecipitates normalized for Raf-1. Fig. 5 shows that the kinase activity of the Y340D/Y341D substituted RBD mutant proteins was the same as RafDD. In contrast, the kinase activity of the S508N mutant was only partially up-regulated, whereas the V589F and S619F Raf mutants displayed no up-regulation of kinase activity in the presence of the activating substitutions. The *lin-45* point mutations had identical effects on 14–3–3 interactions with RafDD as with wild-type Raf-1 (Fig. 5).

Effect of lin-45 Loss-of-function Mutations on the Activity of Membrane-targeted Raf-1—The full activation of Raf-1 following recruitment to the plasma membrane involves interactions with lipids, kinases, and phosphatases. Ras-independent activation of Raf can occur if Raf is targeted to the plasma membrane using the C-terminal K-Ras localization signals (Raf-CAAX) (12). *Lin-45* point mutations were therefore introduced into RafCAAX to determine whether any could be rescued by constitutive plasma membrane localization. Membrane fractions of COS1 cells expressing the constructs were normalized

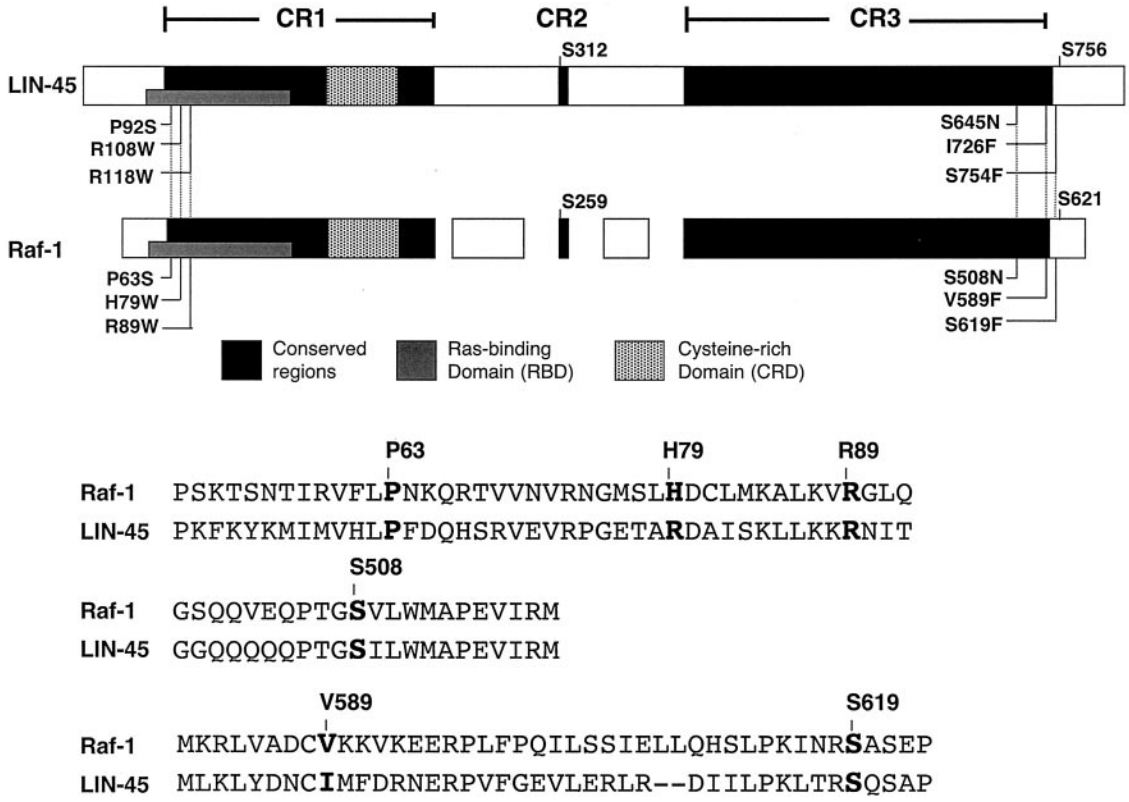

FIG. 1. **Insertions of *lin-45* loss-of-function point mutations into mammalian Raf-1.** Amino acid substitutions were introduced into FLAG-Raf (Raf-1 with an N-terminal FLAG epitope tag) and Raf-GFP (Raf-1 cloned onto the N terminus of GFP). Based on mutations in *lin-45* that cause the substitutions P92S, R108W, R118W, S654N, I726F, and S755F, we introduced mutations that change the homologous Raf-1 residues P63S, H79W, R89W, S508N, V589F, and S619, respectively.

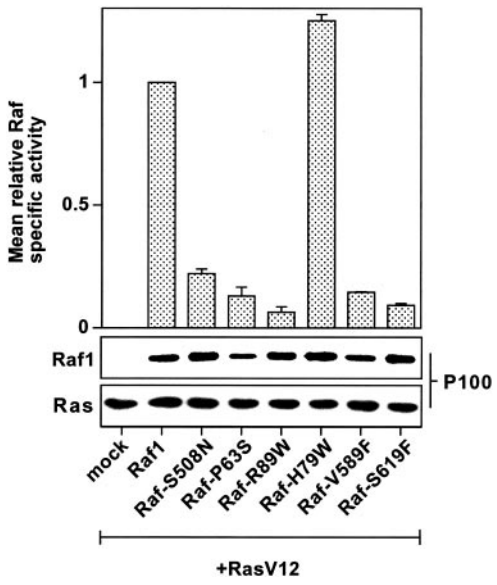

FIG. 2. **Ras-dependent activation of *lin-45* loss-of-function mutations.** COS cells transfected with activated RasG12V and Raf-1 constructs indicated were fractionated and crude membrane (*P100*) fractions were immunoblotted for Ras and Raf-1 (*lower panel*). P100 fractions from each transfection were normalized for Raf-1 content and assayed for Raf-1 kinase activity using a coupled MEK/ERK assay (*upper panel*). The results show mean Raf specific activity from a single transfection assayed in duplicate. Similar results were obtained in three independent COS cell transfections.

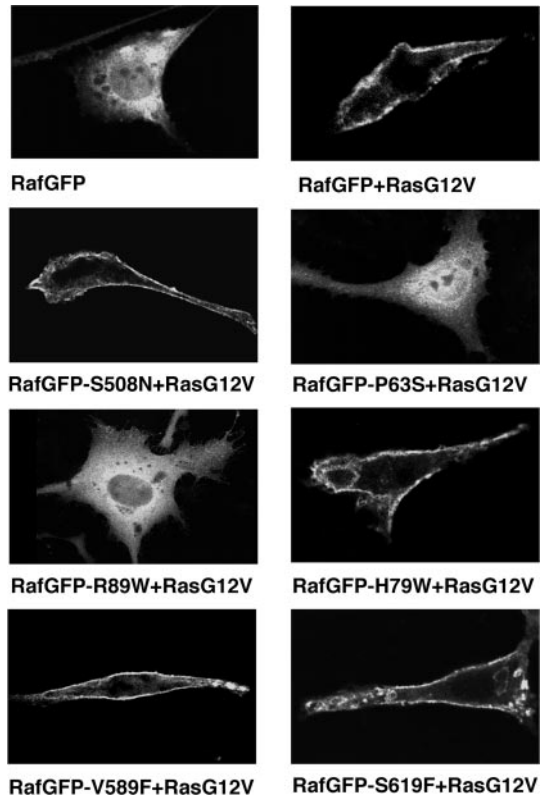

FIG. 3. **Ras-dependent plasma membrane recruitment of *lin-45* loss-of-function mutations.** BHK cells were co-transfected with activated RasG12V and Raf-GFP containing *lin-45* mutations. Raf-GFP proteins were detected by direct fluorescence. Representative cells are shown.

for Raf content, and Raf-1 activity was measured in a coupled MEK/ERK assay. Membrane targeting fully activated the P63S, H79W, and R89W mutants, partially activated the

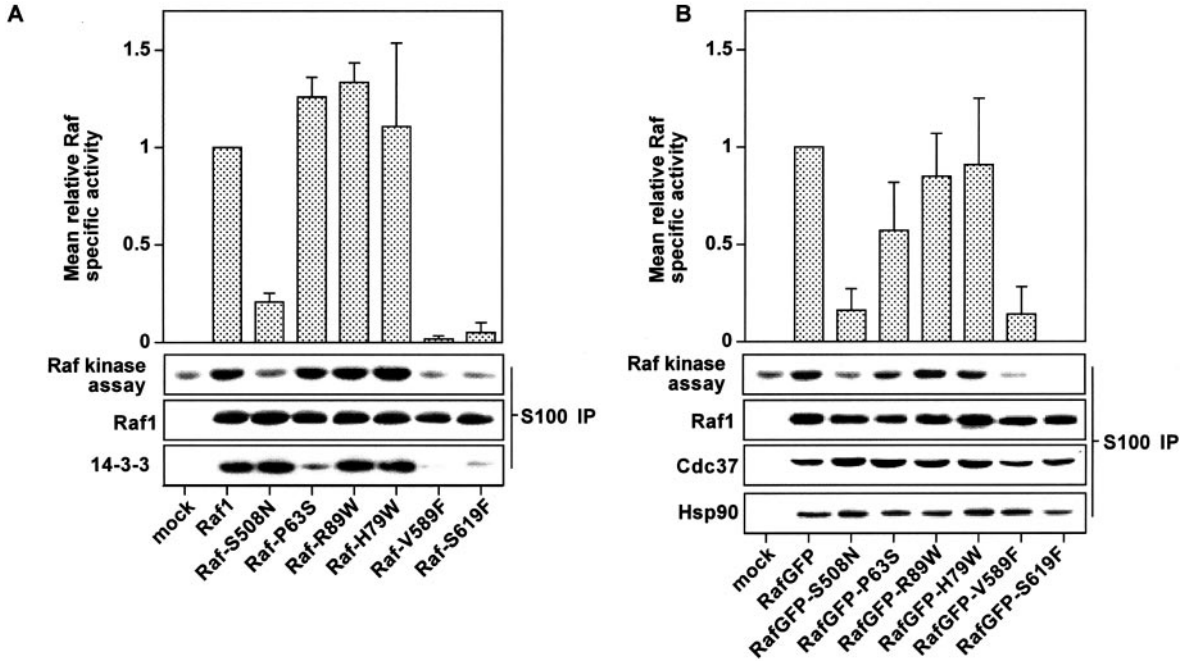

FIG. 4. **Effect of *lin-45* loss-of-function mutations on Raf-1 basal kinase activity and associated proteins.** COS cells transfected with Raf-1 and Raf-GFP constructs containing *lin-45* loss-of-function mutations were fractionated, and the S100 fraction normalized for Raf-1 content. Transfected Raf-1 proteins were immunoprecipitated from the S100 (*S100 IP*) fraction using anti-FLAG (*panel A*) and anti-GFP (*panel B*) antibodies, respectively. Anti-FLAG immunoprecipitates were blotted for Raf-1 and 14–3–3 (*A*, *lower panels*) and anti-GFP immunoprecipitates blotted for Raf-1, Cdc37, and Hsp90 (*B*, *lower panels*). The basal kinase activity of Raf-1 was determined by measuring Raf kinase activity associated with the immunoprecipitates using a coupled MEK/ERK kinase assay performed in triplicate. Typical kinase assays are shown in the *lower panels* of *A* and *B* and the mean of three independent assays displayed graphically in the *upper panels*. Similar results were obtained in three independent experiments.

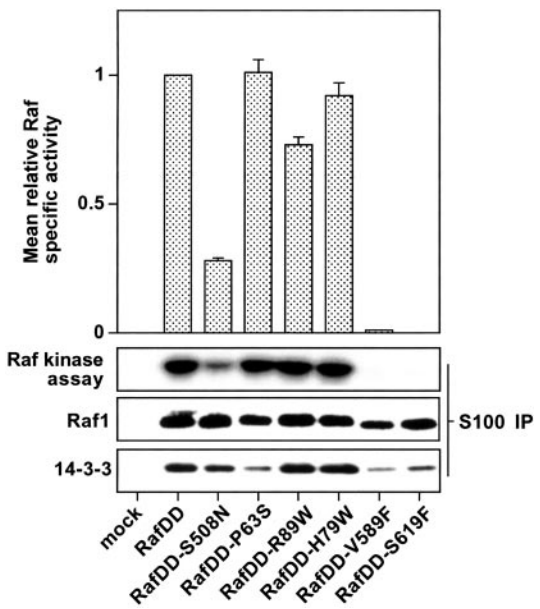

FIG. 5. **Effect of *lin-45* loss-of-function mutations on constitutively activated Raf-1.** Replacement of Raf-1 tyrosines 340 and 341 with aspartic acid (RafDD) activates Raf-1 independently of Ras. *lin-45* mutations were introduced into FLAG-RafDD, and the constructs were expressed in COS cells. Mutant RafDD proteins were immunoprecipitated using anti-FLAG antibodies from the S100 fraction (*S100 IP*). Immunoprecipitates were assayed for Raf kinase activity in a coupled MEK/ERK kinase assay performed in duplicate (shown as a *graph* in *upper panel* with a representative kinase assay gel shown in *lower panel*) then blotted for Raf-1 and 14–3–3 (*lower panels*). Similar results were obtained in three independent transfections.

S508N mutant, and failed to activate the V589F and S619F mutants (Fig. 6*A*). These results, in combination with the data in Fig. 5, suggest that the kinase function of S508N is partially compromised, and the kinase function of the V589F and S619F mutants is highly compromised. This is surprising given that the equivalent mutations to V589F and S619F in *C. elegans lin-45* only caused weak phenotypes. The *C. elegans* genetic analysis nevertheless indicates that a functional kinase domain is necessary for biological function because a nonsense mutation that encodes a LIN-45 protein lacking a kinase domain displayed the strongest phenotype (38).

Although the well characterized coupled Raf/MEK/ERK assay gives an accurate estimate of Raf kinase activity, we wondered whether it accurately reflected the ability of plasma membrane-localized Raf to activate MEK in intact cells. To test this hypothesis, we transfected kinase inactive RafCAAX constructs with the mutations S508N, V589F, and S619F and kinase inactive RafCAAX containing the mutations Y304F/Y341F (RafCAAX-FF) into BHK cells. BHK cells were used for these experiments as they are transfected with greater than 80% efficiency, thus greatly reducing background derived from non-transfected cells. As a control we expressed a "true" kinase-inactive RafCAAX (RafCAAX-KD) that has a K375M substitution in the ATP binding site and is therefore unable to catalyze phosphotransfer reactions (5). MEK phosphorylation was used as the readout for *in vivo* Raf-1 kinase activity. Transfected cells were fractionated into membrane (P100) and cytosolic (S100) fractions then immunoblotted for phospho-MEK (Fig. 6*B*). Strikingly, only RafCAAX-KD was unable to phosphorylate and activate MEK. RafCAAX-FF, RafCAAX-V589F, and RafCAAX-S619F, which had no measurable Raf kinase activity *in vitro*, and RafCAAX-S508N, which had greatly reduced kinase activity *in vitro*, were able to activate MEK to comparable levels as the wild-type RafCAAX *in vivo*.

Ser-508 in Raf-1 and Ser-615 in B-Raf Regulate Raf-MEK Interactions—Although Raf-S508N displayed reduced kinase activity in the constitutive activated Raf-1 background, it was fully competent for MEK activation *in vivo*. Nevertheless, this

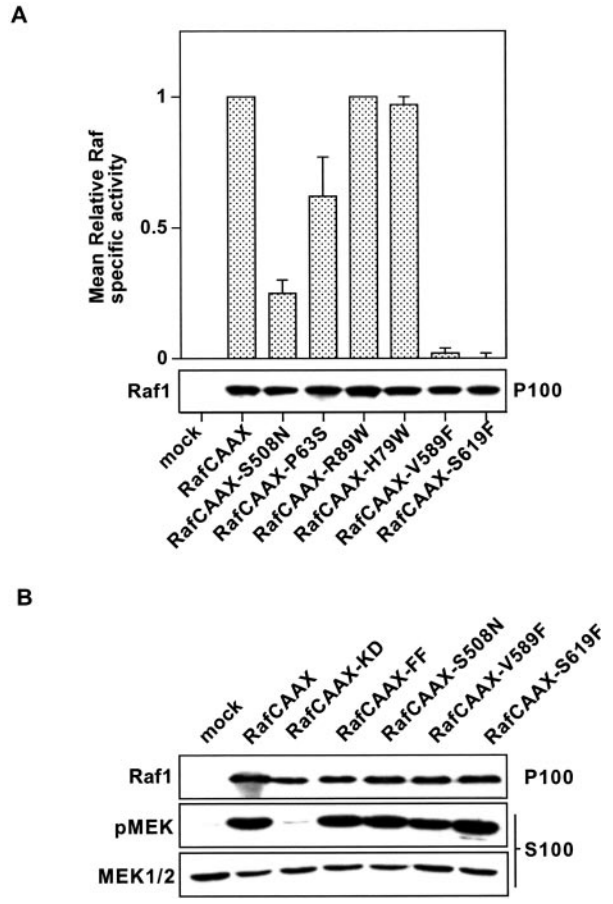

FIG. 6. **Effect of *lin-45* mutations on membrane targeted RafCAAX.** Targeting Raf-1 to the plasma membrane activates Raf-1 independently of Ras. COS cells expressing plasma membrane targeted RafCAAX constructs containing *lin-45* mutations were fractionated into P100 and S100 fractions. P100 fractions normalized for Raf-1 content were assayed in duplicate for Raf-1 kinase activity in a coupled MEK/ERK assay. Identical aliquots of the P100 fractions were then immunoblotted for Raf-1 (*panel A*). Similar results were obtained in three independent transfections. BHK cells transfected with membrane targeted RafCAAX constructs were serum-starved and fractionated. 20 μg of the P100 fractions were immunoblotted for Raf-1 and equivalent portions of the S100 fractions immunoblotted for phospho-MEK (*pMEK*) and MEK1/2 as input control (*panel B*).

mutant displayed the strongest phenotype of all the *lin-45* missense mutations (Table I and Ref. 38). Serine 508 lies within kinase subdomain VIII and forms part of the activation loop of Raf. Phosphorylation on Raf-1 residues Thr-491 and Ser-494 within the activation loop up-regulates Raf kinase activity and is a common mechanism used to control Raf biological function in both *C. elegans* and mammals (42). Phosphorylation within the kinase activation loop can provide catalytic activation through modulation of substrate binding and/or phosphoryl transfer (43). As we have shown that Raf-1 with a S508N substitution can still catalyze the phosphotransfer reaction *in vitro* and *in vivo*, we examined whether this residue modulates substrate binding. Serine is also a potential phosphorylation site, and many protein kinases have a serine or threonine residue at the equivalent residue within the activation loop (44). We therefore substituted serine 508 with aspartic acid in activated RafDD to mimic the negative charge imparted by phosphorylation and examined the effect of this substitution on the ability of Raf-1 to bind to MEK *in vivo* and activate MEK *in vitro*. GFP-tagged RafDD, RafDD-S508N, and RafDD-S508D constructs were expressed in COS cells and the Raf-1 immunoprecipitated with GFP antibody. The immunoprecipitates were divided into two identical aliquots and either assayed for Raf kinase activity or probed for Raf and MEK1/2. RafDD was catalytically active and co-precipitated MEK. In contrast, RafDD-S508N displayed compromised catalytic activity, which correlated with a dramatic reduction in MEK binding. The RafDD-S508D protein displayed a severe defect in catalytic activity and did not co-precipitate any detectable MEK protein (Fig. 7*A*). Thus, the decrease in catalytic activity of the two mutant proteins correlated with a decrease in substrate affinity. To confirm that this residue has a conserved function throughout the Raf kinase family, the equivalent residue was mutated in myc-tagged B-Raf to generate the mutant proteins B-Raf-S615N and B-Raf-S615D. The wild-type and mutant B-Raf proteins were expressed in COS cells, immunoprecipitated with myc antibody, and analyzed for catalytic activity and substrate binding exactly as performed for RafDD (Fig. 7*A*). The B-Raf results were in exact agreement with those obtained for constitutively active Raf-1, showing that decreasing catalytic activity correlated with decreasing substrate binding. These findings strongly support the hypothesis that this residue has a general function in all Raf proteins.

As mutant Raf proteins with compromised catalytic activities can still activate MEK in intact cells, we investigated whether a S508D substitution was sufficient to inactivate Raf *in vivo*. To test this, we expressed constitutively active RafCAAX, kinase inactive RafCAAX-KD, and the mutant proteins RafCAAX-S508N and RafCAAX-S508D in BHK cells and assessed the ability of the mutant proteins to phosphorylate MEK *in vivo*. Strikingly, RafCAAX-S508D was profoundly compromised in its ability to phosphorylate MEK *in vivo* compared with both RafCAAX and RafCAAX-S508N (Fig. 7*B*). This result shows that a negative charge on Ser-508 efficiently down-regulates Raf catalytic activity in living cells.

The Ser-508 Residue Is Critical for Proper Receptor-mediated Raf-1 Activation—Raf proteins require phosphorylation of two conserved sites within the activation loop for full activation (42). We have shown that mutation of the C-terminal serine of the activation loop (Raf-1-S508N, B-Raf-S615N) reduced both catalytic activity and substrate binding. Mutation of this residue may reduce phosphorylation of the activating residues within the activation loop in response to growth factors, or perturb the activation loop such that it cannot form a stable structure after phosphorylation has occurred. One prediction of this hypothesis is that Raf-1 with a S508N substitution will display aberrant activation kinetics in response to growth factor stimulation. To test this prediction, GFP-tagged wild-type Raf-1 and Raf-S508N were expressed in cells arrested at G_0 by serum starvation and then subsequently stimulated by EGF addition. These cells were harvested at time points up to 10 min post-EGF treatment to assess the profile of Raf-1 activation. Wild-type Raf-1 displayed two distinct phases of activation and deactivation within this time frame (Fig. 8). In striking contrast, Raf-S508N, although competent for the first phase of activation at 1 min, was subsequently refractory to the second phase of EGF-receptor activation at 10 min post-EGF (Fig. 8). This result demonstrates that in addition to low kinase activity, the Raf-S508N mutant also possesses an aberrant activation profile in response to growth factor mediated activation.

DISCUSSION

We previously identified a series of point mutations within *C. elegans lin-45 raf* that generated loss-of-function phenotypes and which we scored as weak, intermediate, or strong based on phenotypic severity (Table I). The mutant *lin-45 raf* alleles were assayed in a physiological setting, and for mutations that could be maintained as homozygotes, the mutant protein com-

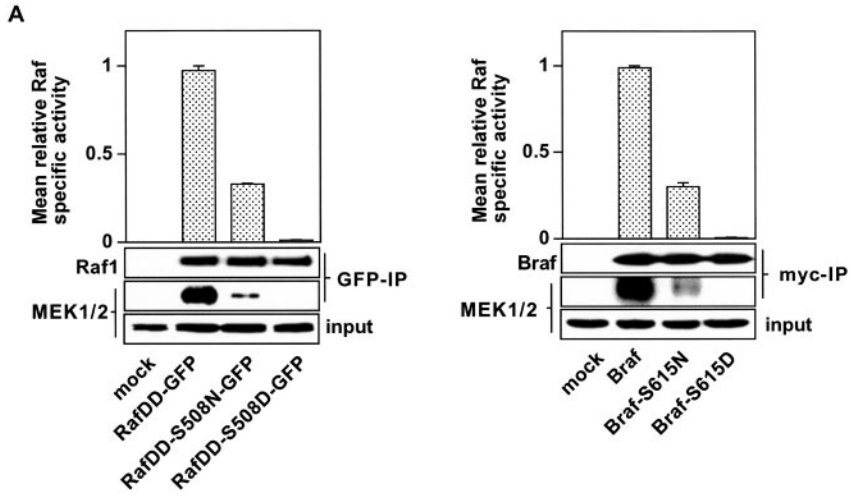

FIG. 7. **The Raf-1-S508/B-Raf-S615 residue mediates substrate binding.** *A*, COS cells expressing GFP alone or GFP-tagged constitutively activated Raf-1 constructs (*left panel*), empty vector or B-Raf constructs (*right panel*) were lysed in buffer B and Raf-1 immunoprecipitated from the cleared lysate using rabbit anti-GFP attached to protein-A agarose (*GFP-IP*) and B-Raf immunoprecipitated using anti-myc attached to protein-G agarose (*myc-IP*). After extensive washing the beads were resuspended in sample buffer and the immunoprecipitated proteins resolved by SDS-PAGE, then immunoblotted for Raf-1 or B-Raf and MEK1/2. 20 μg of the cleared lysate was immunoblotted for MEK1 as an input control. *B*, BHK cells expressing membrane targeted Raf-CAAX constructs were serum-starved then fractionated into P100 and S100 fractions. 20 μg of the P100 fractions were immunoblotted for Raf-1 and 20 μg of the S100 for pMEK and MEK1/2 as input control. Cells transfected with vector alone (*mock*) were included as a negative control.

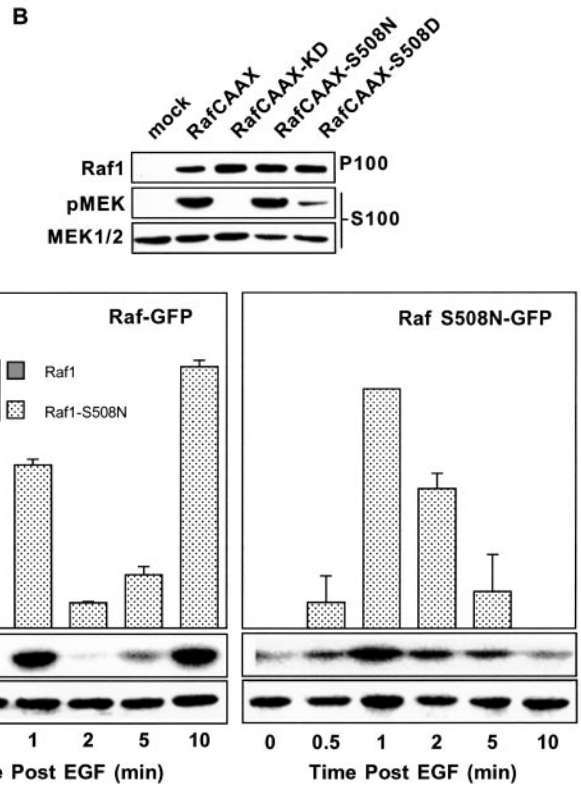

FIG. 8. **The Raf-S508 residue is critical for proper growth factor-mediated Raf-1 activation.** COS cells transfected either with Raf-GFP or RafS508N-GFP, respectively, were pooled after transfection and then divided into 6 identical aliquots to ensure equivalent expression across the EGF time course. At 48 h post-transfection the cells were serum-starved for 18 h then stimulated with 50 nM EGF. Cells were harvested up to 10 min post-EGF addition and fractionated into P100 and S100 fractions. The P100 fractions were then solubilized in buffer A containing 1% Triton X-100, and Raf-GFP immunoprecipitated from the cleared P100 supernatant. After extensive washing, immunoprecipitated Raf-1 was assayed for kinase activity using the *in vitro* coupled assay, and the immunoprecipitates were immunoblotted for Raf-1. A comparison of the relative kinase activity of the Raf-1 wild-type and Raf-S508N at 1-min post-EGF addition is shown as an *inset*.

pletely replaced wild-type LIN-45. This genetic analysis provided strong evidence for the functional significance of particular residues and domains, but it did not reveal the biochemical basis underlying the phenotype caused by the mutant proteins. To understand the biochemical defects caused by these mutations, we engineered them into the equivalent residues of Raf-1 and assayed the mutant proteins for defects in biological and biochemical functions including subcellular localization, kinase activity, and binding to defined protein partners. This combination of approaches is powerful because if a mutation causes a functional deficit and a biochemical defect, this correlation indicates that the biochemical activity is functionally significant.

Three mutations were identified that affect the minimal Raf RBD. The substitutions P92S(*lin-45*)/P63S(Raf-1) and R118W (*lin-45*)/R89W(Raf-1) caused an intermediate loss-of-function in *C. elegans*. Both proteins were refractory to recruitment and activation by Ras-GTP. However, both proteins displayed normal basal kinase activity and could be rescued by constitutive plasma membrane targeting and activating point mutations. These findings indicate that these residues are required for the recruitment of Raf from the cytosol to plasma membrane but do not effect subsequent activation steps. The crystal structure of the Raf-1 RBD in complex with Rap has been solved (45), and Arg-89 of the Raf-1 RBD shown to directly stabilize the interaction through polar interactions and water-mediated contact.

Mutational analyses have also shown that Arg-89 is critical for the interaction between the Raf-1 RBD and the Ras effector domain (15, 16). Our results are in complete agreement with these studies and confirm a physiological role for the Ras-GTP/Raf interaction.

In contrast, the P92S(*lin-45*)/P63S(Raf-1) residue is not directly involved in binding the Ras-GTP effector domain. Rather, this conserved residue lies between two β sheets (B1 and B2) in the Raf-1 RBD (45). It is likely that this residue is folded in a hydrophobic region of the protein and has a role in maintaining the appropriate structure of the Raf RBD. In agreement with this prediction the point mutation P63S completely abrogated Ras-GTP mediated recruitment. The P63S mutant also displayed reduced 14–3–3 binding relative to wild-type Raf-1. We have previously shown that the Raf-1 CRD stabilizes the interaction between Raf-1 and 14–3–3 (23). It is possible that the P63S substitution destabilizes not only the Raf-1 RBD structure but also the adjacent CRD and hence destabilizes the Raf-1/14–3–3 complex. Although these RBD mutants were unable to bind Ras-GTP, they displayed only an intermediate phenotypic strength in the *C. elegans* genetic screens (38). This indicates that in addition to direct contact between the Raf RBD and Ras effector domain, secondary mechanisms must exist that couple Ras-GTP to Raf that can partially compensate for a non-functional Raf RBD *in vivo*. Sur-8 is a scaffold protein found in both *C. elegans* and mammals (46, 47) that enhances ERK1/2 signaling by forming a complex with Ras and Raf (48). In addition, agonist-dependent translocation of Raf-1 to the plasma membrane is mediated primarily through a Ras-independent association with phosphatidic acid (Ref. 49 and references therein). Thus there are at least two mechanisms that might facilitate Raf membrane recruitment independently of the Raf RBD. Our data strengthens the hypothesis that these secondary mechanisms for coupling Ras-GTP to Raf are functionally important in the living animal.

The substitution R108W (*lin-45*)/H79W (Raf-1) caused a weak loss of function in *C. elegans*. Consistent with this weak phenotype, the mutant protein was indistinguishable from wild-type Raf-1 in our assays. It is likely that the substitution causes a biochemical defect that is too subtle to be detected using ectopic expression of the mutant protein. However, we cannot exclude the possibility that this residue may have a function in LIN-45 that is not conserved in vertebrate Raf-1, or that the residue mediates a biochemical function that was not assayed.

Two mutations were identified that affect the protein kinase domain: S645N(*lin-45*)/S508N(Raf-1) and I726F(*lin-45*)/V589F(Raf-1). A point mutation within the C-terminal 14–3–3 binding site was also identified S754F(*lin-45*)/S619F(Raf-1). All three of these mutations had a profound effect on the kinase activity of the Raf-1 protein as judged by the *in vitro* Raf-1 kinase assay. Raf S508N was recruited to the plasma membrane by activated Ras, but was poorly activated. However, Raf S508N was partially activated when placed in constitutively activated Raf-1 (RafDD) or membrane-targeted Raf-1 (RafCAAX). The V589F and S619F Raf-1 mutant proteins were recruited normally by RasG12V but not activated. Because neither of these mutants kinase activity was up-regulated by Y340D/Y341D substitutions or membrane targeting these results show that the I726(*lin-45*)/V589(Raf-1) and S754(*lin-45*)/S619(Raf-1) residues are critical for the kinase function of Raf proteins. Both of these mutant proteins were also defective for 14–3–3 binding. Thorson *et al.* (50) showed that autophosphorylation of Ser-621 is essential for maintaining the interaction of 14–3–3 with the isolated Raf kinase domain. They also described mutations at Ser-619 that prevented 14–3–3 association and destroyed kinase activity of the isolated Raf-1 catalytic domain. Our results confirm that this observation also holds for the full-length Raf-1 molecule.

A critical question that arises from the genetic and biochemical analysis is to what extent Raf biological function is determined by its kinase activity toward MEK. For example, Raf-1 V589F and S619F mutations completely abrogate Raf-1 kinase activity as measured by a standard *in vitro* coupled assay, yet the corresponding mutations in *lin-45* cause weak loss-of-function phenotypes. In contrast the S508N mutant, which displays a reduced but still functional kinase activity *in vitro*, exhibits the strongest phenotype of all the mutants with a single amino acid substitution. The *C. elegans* genetic analysis clearly correlates to kinase activity of the LIN-45 protein, as the alleles *dx19* and *n2510*, which encode proteins that lack the kinase domain, displayed the strongest phenotypes (38). These results are reminiscent of recent studies examining the function of Raf-1 in mouse development. The Raf-1−/− null mouse is nonviable with the embryo showing defects in vascularization and placental development as well as increased apoptosis in many tissues (28, 29, 32). In contrast, knock-in mice expressing mutant Raf-1 Y340F/Y341F in place of endogenous Raf-1 survive to adulthood, are fertile and have an apparently normal phenotype (28). Raf-1 requires phosphorylation of Tyr-340 and/or Tyr-341 for full activation (41, 51). Substitution of these residues to phenylalanine blocks activation of Raf-1 by oncogenic Ras and Src and by constitutive membrane targeting (39). However, mouse embryonic fibroblasts derived from Raf-Y340F/Y341F (RafFF) embryonic tissue showed no increase in programmed cell death relative to wild-type cells and displayed normal proliferation and ERK activation (28). One possible conclusion of the RafFF transgenic study is that the critical biological function of Raf-1 is not dependent on its ability to phosphorylate and activate MEK. In support of this hypothesis an anti-apoptotic, kinase-independent function for Raf-1 has been demonstrated (34). Raf-1 interacts with the pro-apoptotic, stress-activated protein kinase apoptosis signal-regulating kinase 1 *in vitro* and *in vivo*. This interaction allows Raf-1 to inhibit apoptosis independently of the MEK-ERK pathway.

The results presented here, however, provide strong evidence that kinase activity is critical for biological function of Raf proteins in an animal. As Raf-1 is the only Raf isoform ubiquitously expressed in mammals (52), and a large body of evidence suggests Raf-1 kinase activity is indeed essential for its function (reviewed in Ref. 51), caution should be applied before dismissing Raf-1 kinase activity as redundant for its biological function. We have demonstrated that Raf kinase activity as measured by sensitive *in vitro* kinase assays does not reflect *in vivo* kinase activity of Raf proteins. In the definitive experiment, we show that membrane targeted RafCAAX-FF, RafCAAX-V589F and RafCAAX-S619F, all of which are kinase-inactive *in vitro*, efficiently phosphorylate and activate MEK *in vivo*. However, membrane targeted RafCAAX-KD, which cannot bind ATP to perform the phosphotransfer reaction and is truly kinase dead, cannot activate MEK *in vivo*. One possible explanation for the difference between *in vitro* and *in vivo* kinase activity is the presence *in vivo* of potential accessory molecules such as adaptors and scaffolds that facilitate the assembly of enzyme-substrate complexes. Scaffold complexes for MAPK pathways have been well characterized in yeast (reviewed in Ref. 53) and function to increase both the efficiency and specificity of the signaling cascade (54). Several scaffold-like proteins have been identified for the Raf-MAPK cascade (reviewed in Refs. 55–57). Whatever underlying mechanisms are involved, the discrepancy between *in vitro* and *in vivo* Raf-1 kinase activity accounts for the apparently paradox-

ical result of the kinase inactive mutations V589F (*n1924*) and S619F (*n2520*) retaining biological function in the animal model. These results also provide an alternative explanation for the ability of RafFF to substitute for wild-type Raf-1 in mouse genetic studies without needing to revoke the well established kinase function of the Raf-1 protein.

In contrast, the S645N(*lin-45*)/S508N(Raf-1) mutant displayed kinase activity *in vitro*, was fully competent for activation of MEK when targeted to the plasma membrane, yet had the strongest phenotype of all the substitution mutants (Table I and Ref. 38). When this residue was mutated to aspartic acid (Raf-S508D), the mutant protein was severely compromised for *in vivo* MEK activation compared with wild-type Raf and all the kinase defective Raf mutants analyzed (compare Figs. 6*B* and 7*B*). This raises the intriguing possibility that phosphorylation of Ser-508 could negatively regulate Raf kinase activity *in vivo*. Although Ser-508 is not strictly conserved among all kinases, the majority of protein kinases contain either a serine or threonine residue at the equivalent position (44). Ser-508 lies within the Raf activation loop and appears to be critical for the proper function of this domain. Mutation of this residue in Raf-1, or the equivalent residue in B-Raf, also greatly reduced Raf-MEK binding (Fig. 7*A*) and perturbed Raf activation kinetics (Fig. 8). The simplest interpretation of these combined data is that Ser-508 is critical for the structural integrity of the activation loop, and that mutation to asparagine disorders the activation loop so that it occupies the active site, thereby inhibiting substrate binding. The loss of structural integrity caused by the asparagine substitution may also inhibit phosphorylation of the Raf activation loop on the critical activating residues, or prevent the phosphorylated activation loop from folding into the well ordered structure necessary to generate the catalytically active conformation. Both mechanisms are compatible with the observed aberrant activation kinetics of the mutant Raf-S508N protein in response to growth factor stimulation. As the only activating phosphorylation sites found in *C. elegans*, Raf are located within the activation loop (42), perturbing the structure and or function of the activation loop would be expected to severely inhibit the increase of *lin-45* kinase activity in response to growth factor stimulation. We conclude therefore that the strong phenotype displayed by the S645N(*lin-45*)/S508N(Raf-1) mutant in the genetic screens results from a combination of the reduced substrate binding and inability to respond to phosphorylation events in the activation loop, resulting in aberrant activation kinetics in response to growth factor stimulation.

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Identification of Residues and Domains of Raf Important for Function *in Vivo* and *in Vitro*

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